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Negative Immunomodulators – Blunting Immunostimulation and Facilitating Infection

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1. Introduction

The human immune system requires a delicate balance of positive and negative regulators to provide appropriate responses to different stimuli. It is important to control the extent of the response and limit the response at the conclusion of an immune event. The immune system has many negative immunomodulators that assist in this function, including such recently described molecules as PD-1, KLRG1, SOCS1, and Tim-3. These immunomodulators, amongst others, are under direct or indirect control of a group of small non-coding RNAs called microRNAs (miRNAs) that regulate gene expression via degradation or translational repression of their target mRNAs. While these modulators perform a vital function during normal immune responses, they have also been implicated in the pathology of chronic infections, including hepatitis C virus (HCV), herpes simplex virus (HSV) and human immunodeficiency virus (HIV) (Table 1). It is believed that these viruses alter the expression of negative immunomodulators to blunt appropriate immune responses, facilitating persistent viral infection. Because of the role of these modulators and the involved miRNAs, they are being investigated as possible treatments for chronic infections. This review is not exhaustive but highlights several lines of research in regard to negative immunomodulators in innate and adaptive immune responses to stimulation.

| Molecule | Ligand | Expression |
|----------|---------------------------|---|
| PD-1 | PD-L1/PD-L2 | B cells, T cells, DC, NK T cells, Treg, monocytes |
| Tim-3 | Gal-9 | T cells, Treg, monocytes, macrophages, DC |
| KLRG1 | E-cadherin | NK cells, T cells |
| SOCS1 | JAK 2, cytokine receptors | Multiple somatic cells |

Table 1. Negative Immunomodulators implicated in Chronic Infection

2. Negative immunomodulators — The players

2.1. PD-1

The gene for Programmed Death-1 (PD-1) was first discovered in 1992 during experimentation with T cell hybridomas [1]; PD-1 was found to be upregulated in cells undergoing apoptosis, and is a type 1 transmembrane protein 288 amino acids long [2]. It contains an intracellular domain, a transmembrane domain, a stalk approximately 20 aa in length, and an immunoglobulin superfamily domain that contains an immunoreceptor tyrosine-based switch motif (ITSM) and an immunoreceptor tyrosine-based inhibitory motif (ITIM). PD-1 is expressed on T cells, B cells, dendritic cells (DC), natural killer T cells (NKT cells), and monocytes that have been activated, and two ligands, PD-L1 and PD-L2, have been described. PD-1 expression leads to inhibition of cytokine production, including TNF- α , IFN- γ , IL-2, and possibly the cell survival factor Bcl-xL [2, 3]. PD-1 signals have an inverse relationship with TCR stimulation; at lower levels of TCR stimulation, PD-1 signals are stronger; CD28 signaling, however, can overpower the inhibitory effects of PD-1 [4]. When PD-1 binds to its ligand, TCR signaling is decreased, highlighting a function of PD-1 in adaptive immunity. On the cell surface, PD-1 activation induces the phosphorylation of cytoplasmic tyrosines [5, 6]. SHP-2 associates with the PD-1 ITSM, dephosphorylating signals via the P13K pathway and Akt and ultimately decreasing the expression of cytokines such as IL-2, TNF- α , and IFN- γ [2, 5]. Decreases in IL-2 have been associated with cell death, revealing a possible mechanism of function of PD-1 in apoptosis.

2.2. Tim-3

Tim-3 (T cell immunoglobulin and mucin domain 3) was initially found to be expressed on Th1 cells but has since been identified on monocytes, DC, mast cells, and microglia [7-10]. Tim-3 has an IgV domain, a mucin domain, a transmembrane domain, and a cytoplasmic tail [11]. Studies indicate that only the IgV domain binds to the ligand [10]. The most studied ligand of Tim-3 is galectin-9 (Gal-9) [12]. Tim-3 is thought to play a role in both innate and adaptive immune responses. In inactive immune cells, Tim-3 is expressed on DC and functions through Toll-like receptors (TLRs) to facilitate inflammation [13]. However, when Th1 cells are stimulated, Tim-3 expression on those cells is increased and, in the presence of Gal-9, impedes their responses. Tim-3 has also been shown to decrease macrophage activity [13]. Downregu-

lating Tim-3 is associated with an upregulation of the proinflammatory cytokine IL-12, but also IL-10, an anti-inflammatory cytokine, and PD-1 has been inversely correlated with Tim-3 expression. Due to the complexity of Tim-3 interactions, research into the extent and mechanism of the functions of Tim-3 is still ongoing. However, current data suggest Tim-3 is a key regulator in both innate and adaptive immune responses.

2.3. KLRG1

Killer cell lectin like receptor G1 (KLRG1) is found on NK cells and activated T cells [14, 15]. As cells mature and differentiate, they express KLRG1 in higher numbers. KLRG1 also has an ITIM domain, suggesting an inhibitory activity. When bound with its ligand, E-cadherin, KLRG1 has been shown to inhibit degranulation and IFN- γ secretion by NK cells. Furthermore, KLRG1 also inhibits CD4 T cell activity by decreasing CD95 mediated lysis [14, 15]. Henson *et al.* [14, 15] have shown in CD8 T cells that blocking KLRG1 upregulates cyclin D and E and downregulates cyclin inhibitor p27. To prevent T cell activation, both KLRG1 and CD3/TLR need to be stimulated proximally [16], suggesting that both cell signals need to be provided by the same target cell. The KLRG1 ITIM recruits SHIP-1 and SHP-2 phosphatases, facilitating the conversion of PIP3 to PIP2 [17] and thus blocking the actions of PI3K and downstream phosphorylation of Akt [18]. When Akt is not phosphorylated, proliferation is decreased, perhaps by a lack of cyclin D and E and cyclin inhibitor p27 remaining at levels sufficient for inhibition [15].

2.4. SOCS1

SOCS1 is a member of a family called suppressors of cytokine signaling [19]. SOCS members display a wide spectrum of negative cytokine regulation and have a part in preventing harmful cytokine effects in certain organs [20]. SOCS1 is a negative immunomodulator for both cytokine and TLR receptor mediated signaling, and the members of the SOCS family have two key motifs: a SOCS box and a SH2 domain [19]. In addition, SOCS 1 also possesses a kinase-inhibitory region (KIR). The SH2 domain allows SOCS to associate with cytokine receptors and tyrosine kinases, inhibiting cytokine signaling by dampening kinases or making adaptor molecules for receptor sites unavailable [19]. The SOCS box recruits proteins that perform such functions as regulating degradation of proteins bound with SOCS [21]. SOCS1 prefers to use its SH2 domain to bind JAK kinases [20]. SOCS1's inhibitory function comes from reducing JAK kinase activity through the KIR domain and using the SOCS box to degrade JAK kinases. Cytokines such as IL-4, TNF- γ , and some TLR ligands can cause the upregulation of SOCS1 expression [22]. SOCS1 also directly inhibits TLR signaling [21, 22]. The mechanism has not been conclusively demonstrated, but several proposals exist [23, 24].

3. Negative immunomodulators in viral infection – Hepatitis C

Over 180 million people throughout world are infected with Hepatitis C Virus [25]. Of those with an acute HCV infection, 85% become chronic [26]. HCV has developed ways to evade the

host immune system, allowing the virus to persist for prolonged periods of time. HCV is a single-stranded RNA virus with a genome of approximately 9.5 kb. A member of the *Flavivirus* family, this virus's genome codes for a single polyprotein precursor that is cleaved into the proteins necessary for viral propagation. The HCV core protein is of particular interest to researchers, as it is implicated in playing a major role in evading host immune responses [27].

Virus-specific cytotoxic T lymphocytes (CTL) are critical for the elimination of the virus-infected cells. Strong viral specific CTL responses have been demonstrated in those individuals who spontaneously resolve HCV infection [28-30]. In chronically HCV-infected patients, however, virus-specific CTL responses are significantly diminished both in the liver and peripheral blood [31, 32]. Numerous additional studies have reported that impaired virus-specific CD4⁺ and CD8⁺ T cell functions are associated with chronic HCV infection [33-36]. This downregulation of cell-mediated T cell responses may contribute to chronic infection; Th CD4⁺ T cell activation and differentiation may be impaired by HCV [42]. It has been reported that CD4⁺ and CD8⁺ T cell responses, including virus-specific interferon gamma (IFN- γ) production, are severely suppressed in chronically HCV-infected patients through the PD-1 inhibitory pathway [37-39]. Several other investigators have supported these studies, including data suggesting PD-1 blockade can restore T cell functions [40, 41] and that HCV core protein may induce PD-1 upregulation and T cell dysfunction [36].

Additional data on the role of PD-1 in HCV infection has focused on innate immune responses. PD-1 appears to be upregulated in monocytes derived from HCV-infected individuals, and these monocytes are functionally impaired in terms of their ability to express IL-12 [42, 43]. Additionally, both "successful HCV treatment with pegylated interferon/ribavirin as well as blocking PD-1/PDL-1 engagement *ex vivo* were associated with reduced PD-1 expression and improved IL-12 production by monocytes". Studies have also demonstrated that monocyte/macrophages isolated from both healthy and HCV-infected individuals and treated with HCV core protein displayed increased PD-1 expression and decreased IL-12 expression [42, 43].

SOCS1 seems to play an interesting role in chronic HCV infection. Miyoshi *et al.* [44] reported that HCV core protein inhibited SOCS1 expression and Yoshida *et al.* [45] reported SOCS1 gene methylation in hepatitis C patients. These experiments were however performed with hepatocellular carcinomas (HCCs). SOCS1 has been demonstrated to suppress liver cell proliferation [46, 47] and has been implicated as a tumor suppressor, consistent with findings that SOCS1 is downregulated in HCCs [19]. Frazier *et al.* [25] have performed experiments with healthy T cells and found SOCS1 to be upregulated in the presence of HCV core protein. These results suggest HCV core protein increases expression of SOCS1 in T cell populations, decreasing immune responses through the pathways described above; moreover, SOCS1 appears to be downregulated in HCCs in the presence of HCV core protein, altering its role in tumor suppression and liver cell regeneration and further contributing to liver pathology during infection. In innate immune responses, Monocytes isolated from both healthy and HCV-infected individuals and treated with HCV core protein displayed increased SOCS1 expression and decreased IL-12 expression [43]. PD-1 and SOCS-1 were found to associate by co-immunoprecipitation studies, and either blocking PD-1 or silencing SOCS-1 in M/M ϕ led to activation of STAT-1 during TLR-stimulated IL-12 production. Silencing SOCS-1 expression

using siRNAs increased IL-12 expression and inhibited PD-1 up-regulation, suggesting crosstalk between the immunomodulators [43].

KLRG1 has been shown to be upregulated in acute hepatitis C infection in T cells but has a relatively low expression in chronic HCV patients [48]. These investigators suggested that, as the number of TCR-triggering events increases, so does the probability that a T cell will express KLRG1. This implies that TCRs are more frequently stimulated in acute HCV infections than chronic infections. Recent studies in NK cells demonstrated that individuals with chronic HCV infection display a reduction in NK cell number and function concomitant with a significant increase in KLRG1 expression. KLRG1 expression was associated with increased NK cell apoptosis and a diminished capacity to undergo cell activation and produce IFN γ , phenomenon; this could be reversed by KLRG1 blockade [49].

Tim-3 has been studied in individuals with HCV infection and found to have a role in both innate and adaptive immune responses. Patients who have a high level of Tim-3 early in infection are more likely to become chronically infected than those with normal levels of Tim-3. The individuals exhibit a higher percentage of CTLs that express both Tim-3 and PD-1 [50]. Tim-3 blockade restores proliferation, cytotoxicity, and killing of HCV-expressing hepatocytes, and blocking PD-1 in the same cells has an even greater effect in most subjects [50].

Tim-3 expression is also up-regulated on Foxp3⁺Tregulatory cells (Tregs), which could be induced *in vitro* by incubating purified healthy CD4⁺T cells with HCV-expressing hepatocytes. HCV-infected hepatocytes expressed higher levels of Gal-9 and TGF- β , and drove conventional CD4⁺T cells into CD25⁺Foxp3⁺Tregs that express TGF- β and IL-10 [51, 52]. Additionally, recombinant Gal-9 protein transformed TCR-activated CD4⁺T cells into Foxp3⁺Tregs in a dose-dependent manner and blocking Tim-3/Gal-9 ligations abrogated HCV-mediated Treg induction [52].

In innate immune responses, Tim-3 expression was up-regulated on monocytes in HCV-infected individuals and this expression was inversely correlated with IL-12 expression [13, 53]. Tim-3 blockade significantly improved HCV-mediated IL-12 suppression, reduced HCV core-mediated expression of the negative immunoinhibitors PD-1 and SOCS-1, and increased STAT-1 phosphorylation in monocyte macrophages in these studies. Figure 1 represents a potential model for the interplay between the negative immunomodulators during HCV infection.

4. Negative immunomodulators in viral infection— Human Immunodeficiency Virus (HIV)

HIV is a retrovirus with an envelope and is a single-stranded RNA virus. Three enzymes, reverse transcriptase, protease, and integrase, are present in the core of the virus. These proteins are the target of many antiretroviral drugs used for HIV therapy. The HIV pandemic has spurred much of the research into negative immunomodulators, which appear to be involved in many aspects of HIV-mediated immune dysregulation.

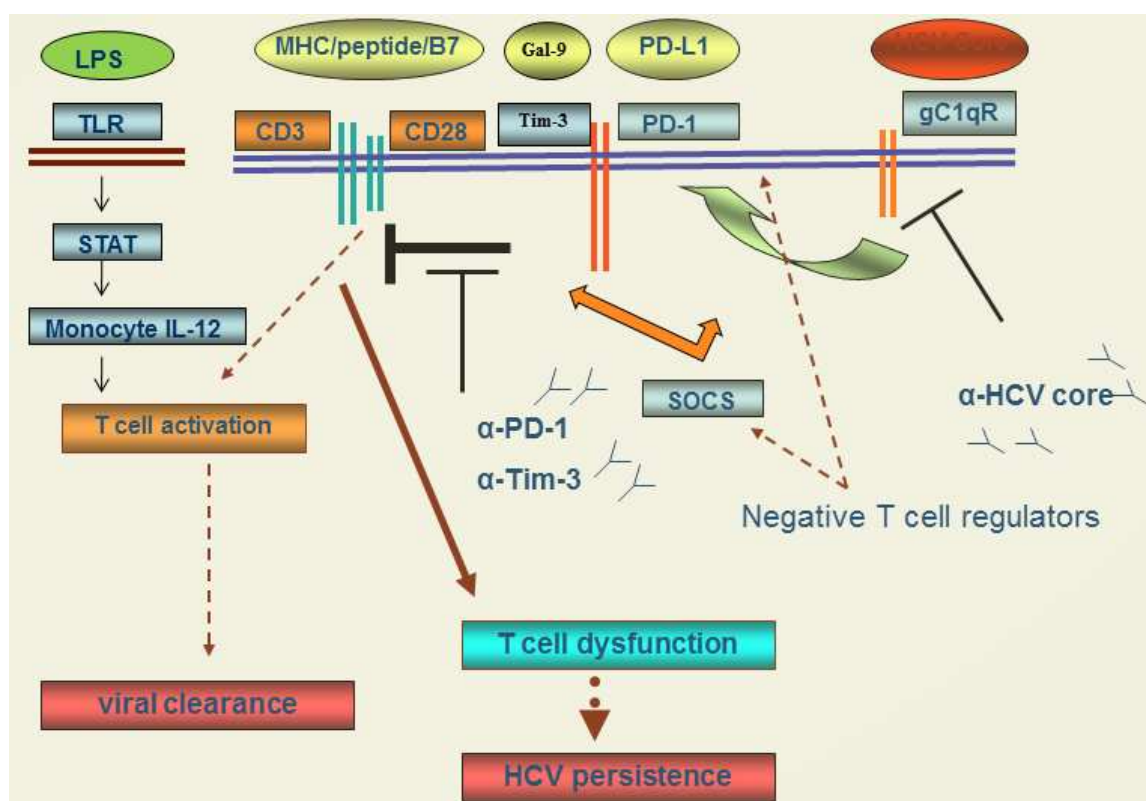


Figure 1. Model for the role of negative immunomodulators during chronic HCV infection.

KLRG1 has been shown by Ibegbu *et al.* [54] to be upregulated on CD8⁺ cells in HIV positive patients. KLRG1 and its ligand, E-cadherin, decreased the cytotoxicity of HIV⁺ CD8⁺ cells [55]. As noted previously, KLRG1 has also been shown to be co-expressed with other inhibitory molecules such as PD1 in chronic infections such as in HCV infected cells [55]. Chronic latent infections such as Epstein-Barr virus and cytomegalovirus also increase KLRG1 expression, while acute viral infections such as influenza do not [30]. This suggests the persistent antigen stimulation in chronic infections may lead to immune senescence through molecules such as KLRG1, making this cell signal a potential target for therapeutic treatments.

SOCS1 expression is increased in HIV infected cells [56]. SOCS1 may aid in HIV particle maturation and infectivity by interacting with the HIV-1 p55 Gag protein. Viral protease cleaves Gag into domains key to viral replication; SOCS1 promotes the stability of Gag, and the SH2 domain may be of key importance in HIV-1 Gag binding [56]. Other SOCS proteins such as SOCS3 do not increase viral replication, suggesting the role of SOCS1 is unique amongst the proteins with a SOCS box domain.

HIV exposures causes increased expression of PD-1 [57], and PD-1 expression on HIV specific CD8⁺ cells correlates with HIV viral load [58], implying antigen signal strength leads to expression of PD-1 and exhaustion [59]. An HIV protein, Nef, was shown to use a MAPK-dependent pathway to increase the expression of PD-1 [60]. PD-1 has been demonstrated to impair the function of cytotoxic T lymphocytes [59], contributing to the decreased immune response characteristic of HIV infection. However, controlling the viral load through tradi-

tional treatment leads to a decrease in PD-1 expression [61]. Blocking the binding of PD-L1 to PD-1 in CTLs has led to an increase in CTLs, which may contribute to a stronger adaptive immune response. These data suggest PD-1 could be a potent target for therapies for HIV. However, PD-1 may protect the vascular system from severe immunological responses during acute infections, with potential difficulties with blocking PD-1 pathways *in vivo* [62, 63].

In HIV-infected patients, Tim-3 expression is increased on T cells [64-66]. The binding of Gal-9 to Tim-3 has been demonstrated to render them less susceptible to HIV-1 infection and replication. The Gal-9/Tim-3 interaction on activated CD4 T cells leads to down-regulation of HIV-1 coreceptors and up-regulation of the cyclin-dependent kinase inhibitor p21 [67]. During the chronic stage of HIV, this interaction can lead to T_H1 and T_C1 cell exhaustion [68]. Thus, T cells expressing Tim-3 during chronic infection have an impaired ability to function properly and to increase in number [64]. CD8⁺ cells that are Tim-3 negative have been shown to have a better ability to degranulate than Tim-3⁺CD8⁺ cells. Blocking Tim-3 pathways in Tim-3⁺ cells has been shown to increase perforin release, increasing cytotoxic ability and the acquired immune response [68]. These data suggest blocking Tim-3 may prove to be a potent therapeutic technique in the treatment of chronic HIV infection.

5. Negative immunomodulators in viral infection — Herpes simplex virus

The viral family *Herpesviridae* contains linear dsDNA, and HSV is a member of the subfamily *Alphaherpesvirinae*. These viruses lyse infected cells and grow rapidly; frequently, herpes viruses establish chronic subclinical infections in nerve ganglia that can develop into recurrent acute infections. Chronic infection by HSV leads to up-regulation of negative immunomodulators in a manner similar to HCV and HIV chronic infections. Maheller *et al.* [69] noted that SOCS1 is up-regulated during infection by HSV used in gene therapy experimentation, and this up-regulation of SOCS1 aided in viral replication. Because of the role SOCS1 plays in the JAK/STAT pathway, inhibition of this pathway may be what facilitates viral replication. It has also been demonstrated that SOCS3 is required for efficient viral replication of some strains of HSV [70]. This evidence suggests SOCS family cytokines have an important role in viral replication and they warrant further research.

During chronic HSV infections, PD-1 levels are elevated on CD8⁺T cells [71], perhaps due to frequent antigen stimulation leading to exhaustion. Murine CD8⁺T cells infected with HSV and expressing increased levels of PD-1 have been shown to produce less cytokines, such as IL-2, INF- γ , and TNF- α , than T cells that express less PD-1. After murine HSV infection, PD-1's ligand, PD-L1, has been found to be upregulated on dendritic cells [71]. It is thought PD-L1 on DCs interacts with PD-1 on CD8⁺T cells to decrease the immune response in chronic viral infections. Some studies support the possibility of blocking PD-1/PD-L1 interactions to increase immune responses to latent HSV infection [71]. Bryant-Hudson and Carr [72], however, reported an increase in viral load in ganglia and murine cases of keratitis when PD-1/PD-L1 interactions were blocked during acute infections. The pathology associated with keratitis may result from innate immune responses, such as inflammation due to cytokines

such as IF- γ [73, 74]. Bryant-Hudson and Carr also hypothesized that PD-L1 may disrupt proper antigen presentation through the down-regulation of costimulatory molecules such as CD80/CD86. The different findings in acute versus latent infections highlight the complex role of many immunomodulators and the difficulties faced in manipulating these molecules in therapeutic settings.

Gal-9 is up-regulated in the presence of both latent and acute HSV infection [75], and the majority of CD8⁺T cells in neural ganglia infected by HSV express Tim-3. This suggests a role for Tim-3 expression in HSV infection. Predictably, it has been demonstrated that Gal-9/Tim-3 interactions decrease the efficiency of CD8⁺T cells in HSV infected neural ganglia [76]. Furthermore, Gal-9/Tim-3 interaction on CD8⁺T cells increases their apoptosis [77]. Reddy *et al.* [75] have supporting evidence that Tim-3 up-regulation facilitates HSV latency. However, in murine HSV keratitis, the immunoinflammatory-mediated pathology of the cornea is increased when Gal-9 is blocked with mAb [76]. In these studies, at least 50% of T cells associated with murine keratitis express Tim-3. Interestingly, additional Gal-9 decreases the severity of ocular lesions, presumably through decreasing the inflammatory response.

6. MicroRNAs in negative immune regulation

Micro RNAs (miRNAs) have been under extensive research as molecular regulators of negative immunomodulation, with well-described roles in hematopoiesis, inflammation, cancer and many other pathological processes [78, 79]. MicroRNAs are a relatively newly discovered class of non-coding RNAs that function as regulators of gene expression, either by transcriptional inhibition or mRNA degradation. They demonstrate effects either through their loss of function or overexpression, leading to pathologic responses that affect diverse cellular processes including cell differentiation, proliferation, and apoptosis. The effective recognition of viral infection and subsequent triggering of antiviral innate immune responses is controlled by multiple regulators, including miRNAs. A panel of miRNAs has been identified, including miR-124, miR-125, miR-223, miR-155, and miR-146, amongst others, and the role of individual miRNAs has been described in relation to specific pathologic process.

6.1. miRNAs in chronic viral infection — Hepatitis C (HCV)

miR-146a was initially identified during efforts to find miRNAs that are important in the innate immune response to microbial infection. These initial studies confirmed miR-146a as a transcriptional target of NF- κ B, and it was thought to have a role as a feedback inhibitor of NF- κ B activation. It is highly expressed in mature immune cells, including DC, peritoneal macrophages, granulocytes and splenic B and T cells [80]. Recent studies revealed that Tim-3 expression on T cells is regulated by the Th1 lineage determining transcription factor T-bet (Anderson *et al.*, 2010), which is translationally regulated by miR-146a. Several studies link dysregulation of expression of several miRNAs to HCV and/or its related immunologic phenomenon [81-86].

6.2. miRNA in chronic viral infection — Human Immunodeficiency Virus (HIV)

There are two functional miRNAs, miR-TAR-5p and miR-TAR-3p, which form the transactivating response (TAR) element of human immunodeficiency virus type 1 (HIV-1). Ouellet et al. [87] reported recently that TAR miRNAs derived from HIV-1 can incorporate into host effector Argonaute protein complexes, which is required if these miRNAs are to regulate host mRNA expression. Bioinformatic predictions and reporter gene activity assays identified regulatory elements complementary and responsive to miR-TAR-5p and miR-TAR-3p in the 3' untranslated region (UTR) of several candidate genes involved in apoptosis and cell survival. These include Caspase 8, Aiolos, Ikaros and Nucleophosmin (NPM)/B23. Analyses of Jurkat cells that stably expressed HIV-1 TAR or contained a full-length latent HIV provirus suggested that HIV-1 TAR miRNAs could regulate the expression of genes in T cells that affect the balance between apoptosis and cell survival.

6.3. miRNA in chronic viral infection — Herpes Simplex Virus (HSV)

One of the characteristics of Herpesviruses is their ability to maintain life-long latent infections in their hosts. As reported by Umbach et al. [88], herpes simplex virus 1 (HSV-1) establishes latency in neurons of sensory ganglia, where the only abundant viral gene product is a non-coding RNA. HSV-1 expresses at least two primary miRNA precursors in latently infected neurons that may facilitate the establishment and maintenance of viral latency by post-transcriptionally regulating viral gene expression. These include the latency associated transcript (LAT) [89, 90], which functions as a primary miRNA precursor that encodes four distinct miRNAs in HSV-1 infected cells. One of these miRNAs, miR-H2 3p, is transcribed antisense to ICP0, a viral immediate-early transcriptional activator thought to play a key role in productive HSV-1 replication and reactivation from latency [91].

7. Conclusions

The negative immunomodulators described herein are the targets of researchers attempting to find treatments for chronic diseases. However, the multi-faceted role of many of these molecules makes it difficult to assign specific functions and these immunomodulators likely function in a complex and often interconnected molecular system. The delicate balance of “on” signals and “off” signals within the immune response leads to challenges for this area of research, but the recent strides made in negative immunomodulation are promising.

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